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The Effects of Jasmonic Acid and Chemicals in the JA Pathway on the Defense Systems and Gene Expression in Moss, Physcomitrella patens and Amblystegium serpens

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The Effects of Jasmonic Acid and Chemicals in the JA Pathway on the Defense Systems and Gene Expression in Moss, *Physcomitrella patens* and *Amblystegium serpens.*

A Thesis

Presented to the Department of Biology

College of Liberal Arts and Sciences

And

The Honors Program

Of

Butler University

In Partial Fulfillment

Of the Requirements for Graduation Honors

Allison Shanks

May 6, 2014

DEDICATION

This thesis is dedicated to Dr. Nat Hauck for being my mentor and assisting me through my research. I would also like to dedicate this to my family who has always been a loving support system for me.

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ABSTRACT

Systemic acquired resistance (SAR) is a defense system used by plants that results in increased resistance to future pathogen infection following an initial pathogen exposure. SAR in vascular plants has been well documented; however, a similar defense system has only recently been documented in non-vascular plants. It is believed that chemicals in the jasmonic acid (JA) pathway are able to activate the SAR response in vascular plants. The non-vascular plant, *Amblystegium serpens,* will be used as a model to test if SAR is triggered by JA and two other molecules in the JA pathway, 12-oxo-phytodieonic acid, and methyl jasmonate. To test whether or not SAR has been put into effect, *A. serpens* will be inoculated with the fungal pathogen *Pythium irregulare* after initial hormone application. A second moss species, *Physcomitrella patens,* will be used for analysis of gene expression after fungal elicitor or hormone application. This research will further develop our understanding about the systemic defense response in non-vascular plants, and will aid in our understanding of the evolution of this important mechanism.

ABBREVIATIONS

HR: Hypersensitive Response

SAR: Systemic Acquired Resistance

PR: Pathogenesis related

P. patens: Physcomitrella patens

A. serpens: Amblystegium serpens

A. thaliana: Arabidopsis thaliana

JA: Jasmonic Acid

MeJA: Methyl Jasmonate

OPDA: 12-oxo-phytodienoic acid

SA: Salicylic Acid

PDA: Potato Dextrose Agar

PCR: Polymerase Chain Reaction

qRT-PCR: Quantitative Real Time PCR

CHS: Chalcone synthase

LOX: Lipoxygenase

AOC: Allene Oxide Cyclase

AOS: Allene Oxide Synthase

PAL: Phenylalanine ammonia-lyase

INTRODUCTION

Importance of Plant Defense Systems

Plants have evolved defense systems in order to protect themselves from bacteria, fungi, and predators. These defenses, including both physical structures and chemicals, inhibit infection and prevent the spread of disease in plants (Freeman and Beattie 2008). Plants do not have an immune system homologous to the one seen in mammals, with antibodies and specialized cells that ward off pathogens. Instead, once a pathogen has been introduced into the plant, the plant must utilize toxic chemicals and hormone signals in order to impede pathogen proliferation and spreading. To keep the pathogen in one contained area, the plant can often sacrifice the infected tissue and protect the rest of the plant. These chemicals and hormones have been effective and valuable methods of protecting plants from pathogens, but we do not fully understand the defense mechanisms or the signals that initiate these responses (Loake and Grant 2007). The genes that play pivotal roles in these defense systems are now being identified and new pathways to elicit these defenses are being found. Ongoing research is giving us a more complete picture of the relationship between pathogens and plants.

Overview of Plant Defense Systems in Vascular Plants

Vascular plants have non-induced physical defenses, such as the cuticle and cell well, and in some species, thorns and spines (Freeman and Beattie 2008). These lines of defense are always available and continuously protecting the plant from herbivore and pathogen attack. Physical, non-induced defenses are the primary response to herbivores, pathogens, and parasites in order to prevent them from entering the plant. These structures keep pathogens away from vital plant structures, such as the vascular

system. If a pathogen infiltrates the vascular system, it has access to the entire plant through the veins. Once the pathogen has invaded the plant, induced defense systems are necessary to assist in controlling pathogen proliferation. There are two main induced defenses, the hypersensitive response (HR) and systemic acquired resistance (SAR). HR is an immediate and localized response to pathogen exposure (Morel and Dangl 1997), whereas systemic acquired resistance (SAR) provides pathogen resistance to all areas of the plant (Ryals 1996).

PR and Other Defense Genes in Vascular Plants

Pathogenesis related (PR) genes are induced by fungal, bacterial, or viral infection or by insect-induced damage (Van Loon 2006). There are 17 different families of PR proteins that have been identified and classified so far (Ebrahim 2011, Table 1). The PR genes (Table 1) have a wide range of functions, including cell wall degradation, proteinase inhibitors, peroxidases, defensins, lipid-transfer proteins, and other various defense related functions. Five important classes of PR genes (PR-2, PR-3, PR-4, PR-8, and PR-11) function as chitinases and β-glucanases that degrade various fungal cell walls. These proteins all induce resistance to bacteria or fungi by inhibiting growth of the infecting agent. PR proteins accumulate locally to induce HR and also are expressed throughout the plant, which helps initiate SAR (Van Loon 1999). Another important gene involved in plant defense is chalcone synthase (CHS). CHS functions as the first step in synthesizing flavonoids in plants (Fuessner and Wasternack 2002). It is often used as protection against UV damage and is expressed in response to fungal pathogens (Oliver 2009). CHS is part of a pathway in plants that eventually leads to the production of chalcones, which function as a defense mechanism (Fuessner and Wasternack 2002).

Table 1: PR Protein Families and their Functions. Adopted from Ebrahim, 2011.

PR Family	Function
PR-1	Antifungal
PR-2	B-1,3-glucanase
PR-3	Chitanase type I, II, IV, V, VI, VII
PR-4	Chitanase type I, II
PR-5	Thaumatin-like
PR-6	Proteinase – inhibitor
PR-7	Endoproteinase
PR-8	Chintinase type III
PR-9	Peroxidase
PR-10	Ribonuclease like
PR-11	Chitanase type I
PR-12	Defensin
PR-13	Thionin
PR-14	Lipid- transfer protein
PR-15	Oxalate oxidase
PR-16	Oxalate oxidase –like
PR-17	Unknown

HR in Vascular Plants

HR provides immediate and localized resistance to pathogens after initial exposure. Usually within hours, HR is fully expressed and the effects of this response are measurable (Morel and Dangl 1997). Due to the infecting agents, a cascade of PR genes and other defense related genes are induced, leading to HR. HR is thought to be induced by recognition of the pathogen avirulence gene product and causes chemical changes and increased levels of reactive oxygen species (ROSs) and ions (Morel and Dangl 1997). The HR response is characterized by localized cell death due to ROSs and ion fluxes (Morel and Dangl 1997). The ROSs, specifically the superoxide radical, causes cell death, but as previously stated, this only confers localized resistance. Not only are the infecting agents killed, but the plant tissue also dies due to these ROSs. The rapid ion fluxes cause a swift influx of calcium and efflux of potassium and chloride ions (Morel and Dangl 1997). This causes the cytoplasm to become more basic which is important for inducing cell death. Plants, to prevent universal cell death, also have evolved an anti-cell death pathway. This anti-cell death pathway protects against uncontrolled cell death and inhibits ROSs from killing more cells than necessary in the plant (Morel and Dangl 1997).

SAR in Vascular Plants

SAR provides resistance to all areas of the plant after initial exposure to a pathogen. The plants can detect pathogens through recognizing pathogen activated molecular proteins (PAMPs) that are on the surface of the microorganism (Parker 2003). After the plant detects the presence of the pathogen, a signal is produced that travels throughout the entire plant to all healthy tissue to induce expression of PR and defense genes to protect the plant from subsequent attacks (Tiryaki and Tunaz 2004). It is

thought that the signaling molecules that travel through the plant utilize the vascular system to be efficiently transported throughout all areas. Once SAR is initiated, PR genes are expressed in the plant, which leads to nonspecific disease resistance (Ward et. al. 1991). It has been shown that PR proteins are often associated with SAR (Van Loon 1994). Once expressed, these PR proteins are available in case of future attack so that the pathogen will not survive long enough to be able to infect the plant.

Role of Hormones in Plant Defense in Vascular Plants

Jasmonic acid (JA), salicylic acid (SA), and ethylene are all key plant hormones in inducing both HR and SAR (Loake and Grant 2007). They initiate PR gene expression, which confers resistance to the plant locally and throughout the plant as a whole. SA was originally thought to be the main controlling mechanism behind SAR, but recently it has been shown that other hormones may be playing important roles in initiating SAR (Loake and Grant 2007). The cascade of hormones that are produced after pathogen exposure are what help lead to resistance in the plant. The chemical signals of SAR, SA, JA, and ethylene, are transported through the phloem in vascular plants (Durrant and Dong 2004). Specific genes that encode enzymes needed for the synthesis of the hormones are induced and can be measured during HR or SAR in the plant. Phenylalanine ammonia-lyase (PAL) is a gene initiated during SAR that encodes an enzyme required at an early step in the biosynthesis of SA (Meier 1993). PAL initiates many other genes that turn on defense genes, such as CHS (Meier 1993). The main goal of PAL is to work as one step in the pathway that converts phenylalanine into SA in order to have SA initiate the defense response in plants (Meier 1993). JA has also been shown to initiate SAR in vascular plants.

JA Pathway in Vascular Plants

JA has a similar cascade pathway to SA, which initiates the defense response (Figure 1). After a wound or infection, the plant converts membrane lipids into JA through a series of reactions catalyzed by lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC), as shown in Figure 1. In the JA family, 12-oxo-phytodienoic acid (OPDA) and methyl jasmonate (MeJA), along with JA itself, have been indicated in the defense response in vascular plants (Avanci 2010).

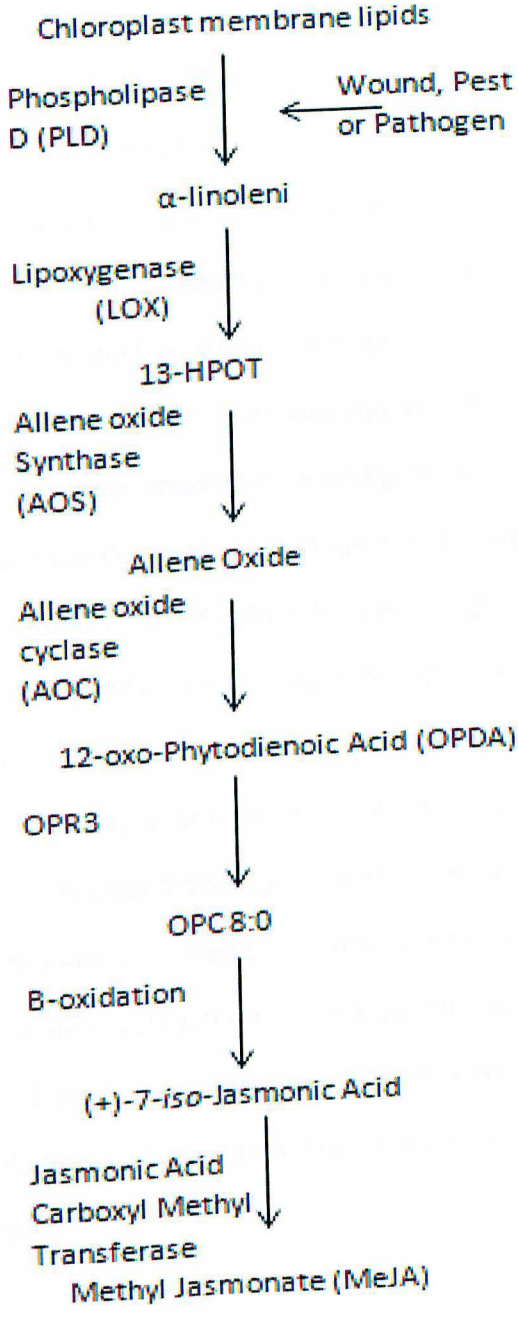


Figure 1: Jasmonic Acid Biosynthetic Pathway in Plants. Upon detection of pathogen, LOX, AOS, and AOC are induced which leads to the eventual production of OPDA, JA, and MeJA.

Plant Evolution

Around 450 million years ago, the first land plants evolved from green algae. These plants were non-vascular so they lacked the means to grow taller, since they had no vascular system to transport water or nutrients. These non-vascular plants, mosses, liverworts, and hornworts, were also known as bryophytes. The lack of a vascular system caused the plants to stay low to the ground where the water and nutrients were. Once plants evolved a vascular system, they were able to transport materials. Complex vascular plants, angiosperms and gymnosperms, evolved from less complex, non vascular plants, as shown in Figure 2. The vascular system evolved during the Silurian period, around 420 to 440 million years ago. Xylem and phloem constitute the vascular system in plants, transporting water and nutrients, respectively. With this new vascular system, plants could grow taller and more complex than before. These plants often relied on water to move sperm until seeded plants evolved. Seeds gave the plants the ability to transport their zygotes while also providing nutrition to the developing plant. Non-flowering, seeded plants are known as gymnosperms. Flowers were later developed which gave plants a reproductive advantage by attracting potential pollinators. Angiosperms, flowering, seeded plants, evolved after gymnosperms. The development of a complex fruit, a mature ovary, often will facilitate in the dispersal of the seeds.

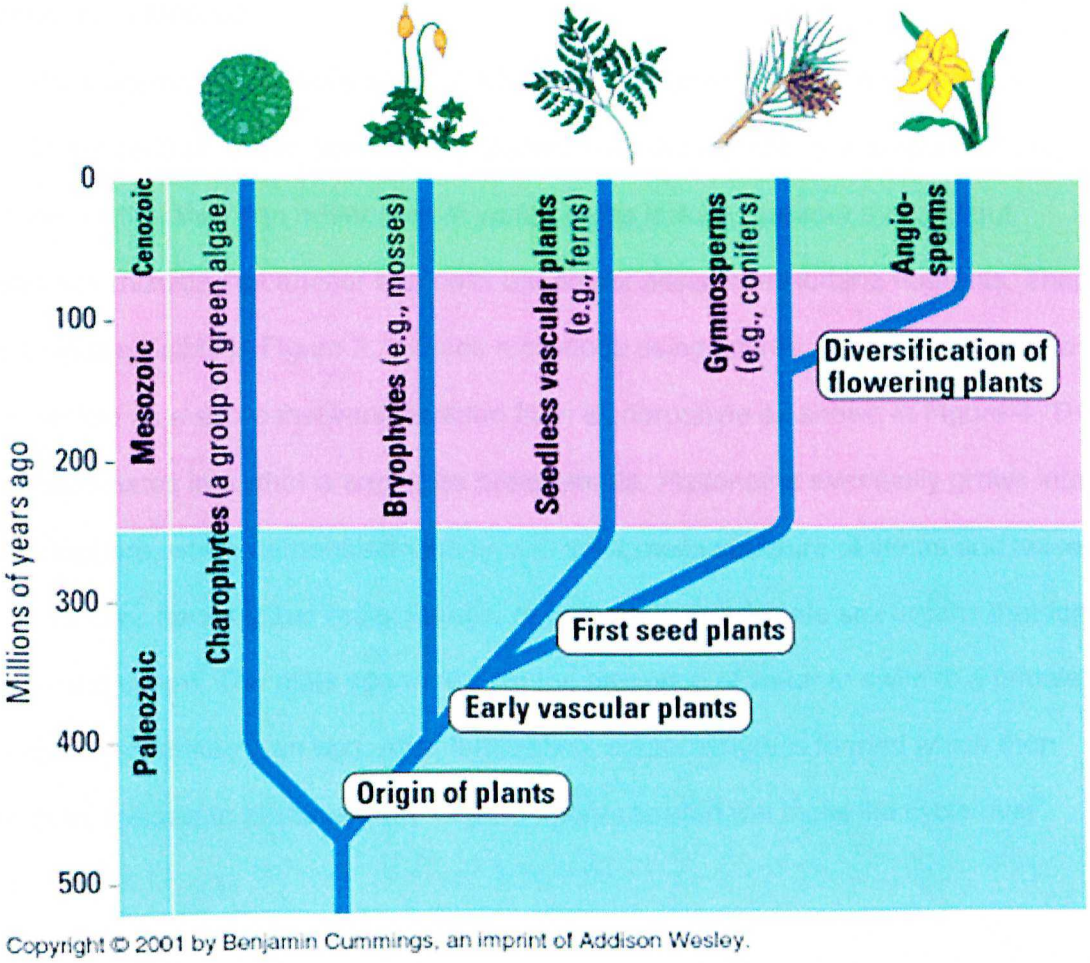


Figure 2: Evolutionary History of Plants. The evolution of plants from green algae involves many major evolutionary events, such as the evolution of a vascular system, seeds, and flowers. This figure shows the pathway of evolution from green algae to flowering plants. From http://ohioplants.org/wp-content/uploads/2012/04/plantkingdom.gif

Morphology of Mosses

Moss plants are generally short, 1-10 cm tall and grow in damp environments. They do not seed or flower, like vascular plants. Their leaves are very simple and are often thin so that they can better absorb water. Moss lacks a true root system, but instead has rhizoids that anchor the moss but do not assist in absorbing nutrients. These structures are visible in Figure 3. Mosses reproduce using spores. They begin their life cycle haploid as a spore that was released from a sporophyte as shown in Figure 4. This spore germinates into what is known as protonemata. Protonema eventually grows into a gametophore, which demonstrate the typical adult moss structure of stems and leaves. Gametophores house either male, female, or both male and female sex organs that form the egg and sperm. The male sperm require the presence of water to swim to a female sex organ and fertilize an egg. After fertilization, a sporophyte is formed which then undergoes meiosis to produce more haploid spores to start the moss life cycle over again.

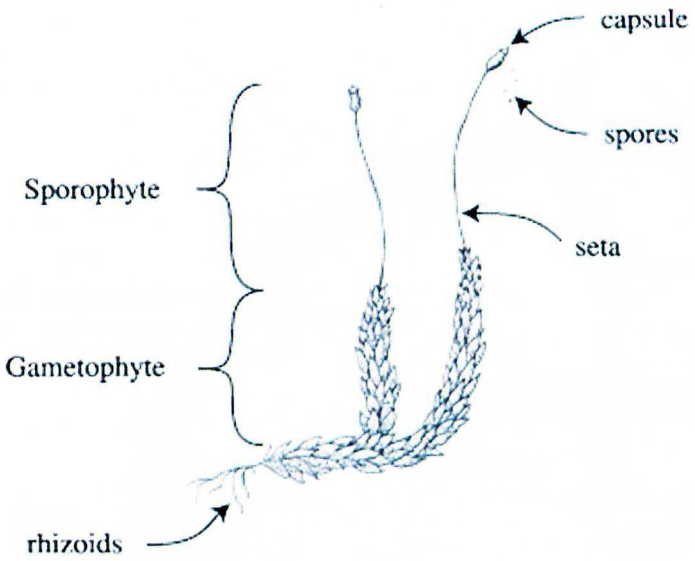


Figure 3: Morphology of a Moss Plant. This figure depicts several reproductively important structures in moss, such as the gametophyte and sporophyte generations. Non-reproductive structures, such as the rhizoids and leaves, are important for basic life functions. From *http://www.kentuckyawake.org/Ferns_mosses_horsetails*

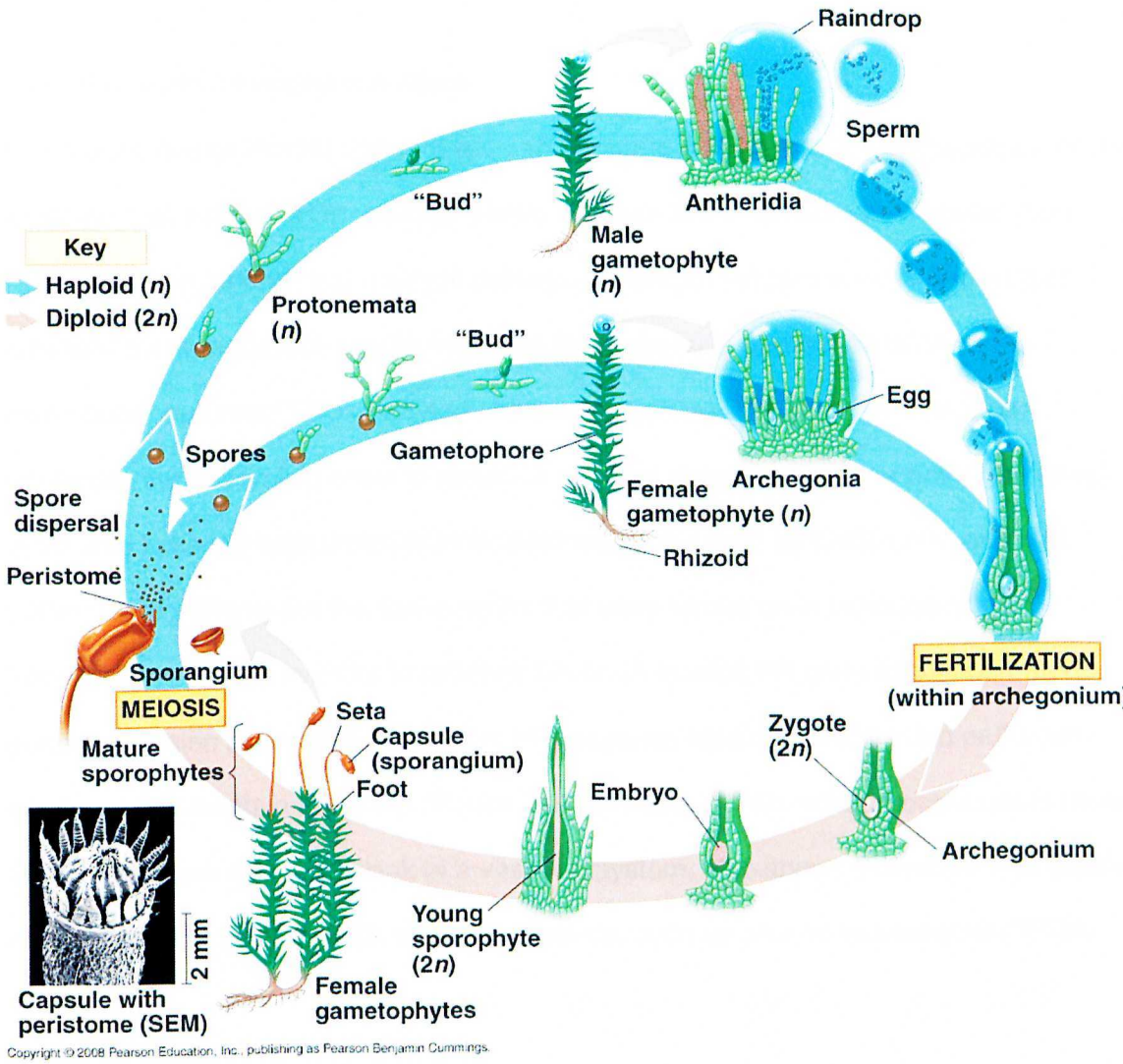


Figure 4: *M*oss Life Cycle. Moss goes through both haploid and diploid stages in its lifecycle. This cycle shows the stages that moss travels through as it develops from a spore to a mature sporophyte. From http://gardeningstudio.com/plants-diagram-2/

Plant Pathogen Interactions in Moss

It has been demonstrated that mosses can generate a HR response to chemicals similar to stimuli that initiate HR in vascular plants. Studies with a model non-vascular plant, *Physcomitrella patens,* and a fungal pathogen, *Pythium irregulare,* have shown that localized plant resistance can be triggered by exogenous application of JA or its immediate precursor, 12-oxo-phytodienoic acid (OPDA) (Oliver 2009). The moss reinforces the cell wall in order to enhance physical defenses to future pathogen attack while also initiating expression of plant defense genes such as CHS, LOX, and PAL (Oliver 2009). These are the same genes that were turned on in vascular plants following an infection in order to produce SA or JA to elicit PR gene expression. PR genes have also been seen during this HR response leading to ROSs and pathogen-mediated cell death in the moss (Ponce de Leon 2007). The moss, thought not to have a SAR response due to the lack of a vascular system, also shows increased expression of chemicals that initiate SAR in vascular plants, such as JA and its precursor OPDA (Oliver 2009).

SAR in Moss

During SAR, either JA or another initiating chemical turns on PR genes (Tiryaki and Tunaz 2004). PR proteins are intended to limit and prevent the spread of pathogen infection. Although this study suggests that JA can induce plant resistance, they were not conducted in a way that allowed isolation of the site of application and a distal site. By isolating the two sites we can determine that application of JA alters the genes expressed at a distal site, lending support to the idea that SAR is utilized by moss. A vascular system was thought to be necessary in order to transport signals that initiate

SAR in plants. Since a moss does not have a vascular system, it was believed that moss could not elicit a SAR response. Moss does produce the hormones, such as JA and SA, which elicit SAR so more research was completed to see if a SAR-like response was available in moss. It was shown that *Amblystegium serpens* has the ability to initiate SAR in response to *Pythium irregulare* (Winter et al. submitted). Winter et al (submitted) were able to show that previous inoculation with the pathogen or treatment with a fungal elicitor (β-glucan) resulted in systemic resistance to future inoculation.

Role of Hormones in Plant Defense in Moss

Future studies are needed to determine the role of hormones involved with SAR in moss. It has been shown that JA and OPDA are both produced by moss in response to pathogens (Oliver 2009), but it needs to be confirmed that these chemicals are triggering SAR and are not just present in the moss. Jasmonic acid and OPDA are precursors of MeJA and can be converted to MeJA during times of stress or when the moss detects a wound (Turner 2002). It has also been thought that SA is an important SAR trigger, so SA must be tested as well to see if it can initiate SAR in moss due to its function in vascular plants.

Thesis Statement

I propose to study the role of JA, OPDA, and MeJA in SAR. I would use the fungus *P. irregulare* to determine if the moss defense system is in place. The specific genes that are turned on with each chemical would then be established through quantitative RT-PCR (qRT-PCR) techniques. I hypothesize that JA, OPDA, and MeJA will induce SAR in the moss *P. patens* and *A. serpens* and that these chemicals will activate PR genes throughout the plant. I expect to find that after the pathogen infects the moss, the genes

regulating the pathway between OPDA, JA, and MeJA are turned on converting more OPDA and JA to MeJA.

Chapter 1: Effects of Exogenous Jasmonic Acid Application on the Growth of the Fungus, *P. irregulare,* and its ability to infect the moss, *Amblystegium serpens*

Introduction:

HR and SAR are both induced defense systems designed to give the plant resistance after a plant identifies a pathogen. HR will confer resistance locally at the site of the infection, while SAR will help protect the whole plant from future infections (Morel and Dangl 1997 and Ryals 2006) It was originally thought that only vascular plants could initiate SAR, due to them having a vascular system to transport chemicals throughout the plant. However, it has been shown that nonvascular plants, such as *P. patens,* have the ability to generate both SAR and HR in response to infection as well (Oliver 2009 and Winter et al, submitted).

It has been shown that JA and chemicals in the JA family induce resistance in moss (Oliver 2009). When JA, OPDA, or MeJA are exogenously applied to moss, not only HR, but also SAR should be initiated. When SAR is initiated due to JA, genes in the JA pathway should also be expressed.

When JA is applied to *P. patens*, both HR and SAR should be initiated leading to resistance to all areas of the plant. In this study, the fungus *P. irregulare* was applied to the moss either with or without pretreatment with JA, with the hypothesis that JA treatment will induce resistance to the pathogen and prevent the fungus from killing the moss. In addition, I aimed to determine if JA directly interferes with the growth of *P. irregulare.*

Methods:

Maintaining and Preparing the Moss and Pathogen

A. serpens was grown on BCD media with micronutrients, according to the instructions by David Cove (2009). The moss was kept at 22°C with 16 hours of light and 8 hours of darkness. *P. irregulare* was kept on PDA media at 22°C. New *P. irregulare* PDA plates were started 24 hours before moss inoculation. *A. serpens* was cut into 2 cm long pieces and transferred to a new BCD plate prior to hormone treatment.

JA Treatment and Inoculation of Moss

3 µL of 50 µM JA, OPDA, or MeJA was applied exogenously to one end of the moss, *A. serpens.* After 24 hours, a 5mm x 5mm PDA plug of *P. irregulare* was placed at either the site of the chemical application or at the distal end of the moss. Plants were observed for 7 days to determine if the fungal treatment was fatal. Additional samples were treated with water to be used as a control.

P. irregulare Growth Rates in Presence of JA Chemicals

100 µL of JA, MeJA, and OPDA, all in 50 µM concentrations, were applied to PDA media. Two replicates of each chemical were done; also two replicates with water as a control were used. A 2cm x 2cm plug of *P. irregulare* was added to each of the plates. Growth of the fungus was measured periodically (every 8-12 hours) to determine the growth rate.

Statistical Analysis of P. irregulare Growth Rates

Using the growth data obtained after growing *P. irregulare* with JA, OPDA, MeJA, and water, graphs were made to determine the rate of growth. The slopes of the lines were representative of the fungal growth rate. T-tests were performed to compare the mean growth rate in the presence of water versus each chemical treatment. Any T-test with a p-value less than 0.05 was concluded to be significant.

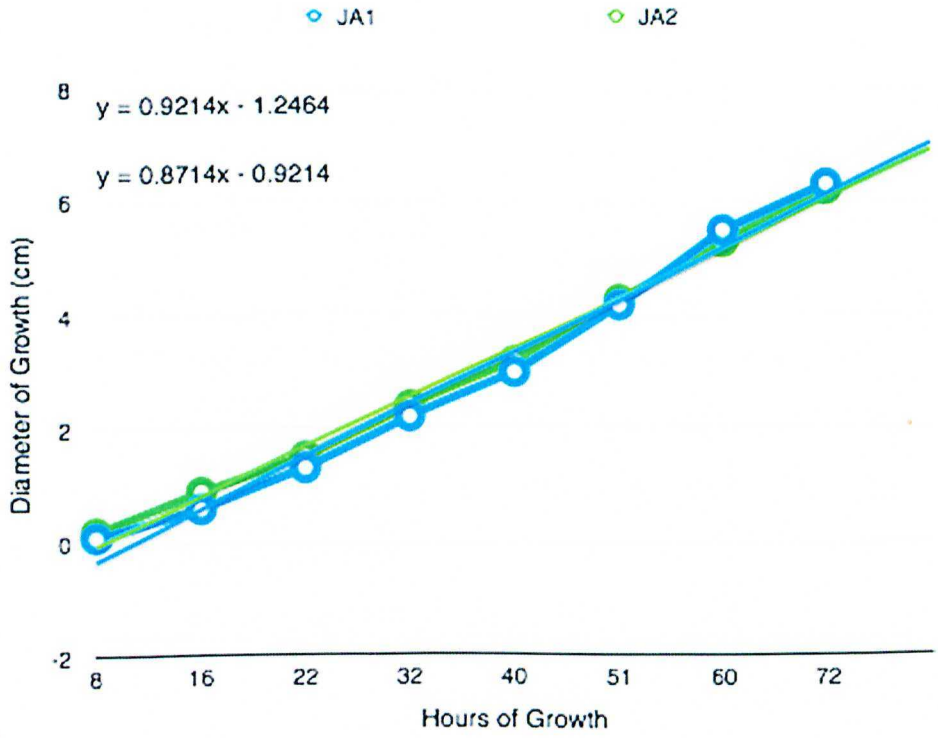
Results:

JA Confers Resistance to A. serpens

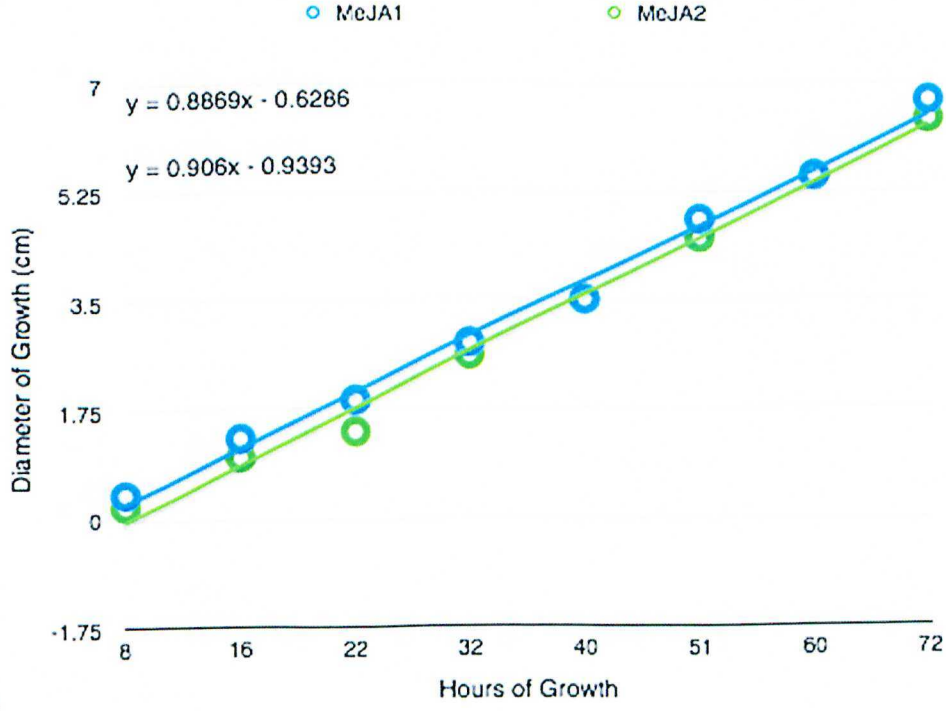
In one trial, all moss samples died regardless of chemical application. There was no phenotypic difference between hormone treated and non-treated control samples.

Growth Rates of P. irregulare in Presence of JA Chemicals

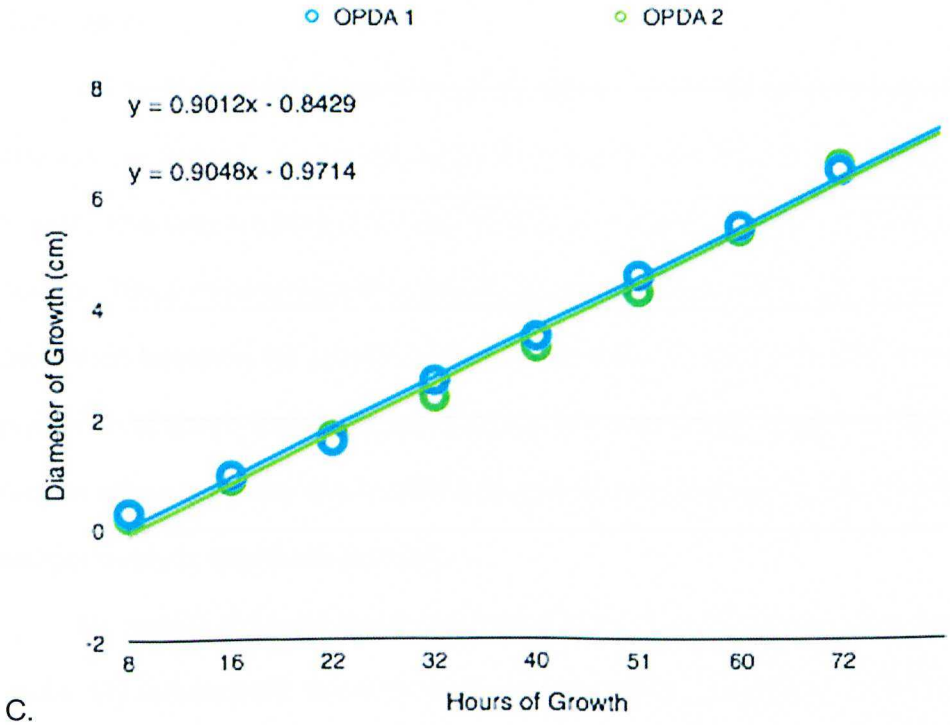
It was shown that none of the JA chemicals inhibited *P. irregulare* growth rates. The growth rates (in cm/hour) for the four conditions with two replicates each were as follows; JA- 0.9214 and 0.8714, MeJA- 0.8869, 0.906, OPDA- 0.9012, 0.9048, and Water- 0.8929 and 0.9167, as shown by Figure 5. T-tests were performed in order to determine whether or not growth was impaired by the presence of the chemicals. The means of the growth for the two replicate plates were used in the statistical analysis and compared to the growth in the presence of water. The T-test with JA and water gave a T-stat of 0.303, a 2 tailed critical value of 4.30, and a 2 tailed p-value of 0.79. The critical value was consistent for all three trials. The T-test for MeJA and water gave a T-stat of 0.547 and a 2 tailed p-value of 0.64. The T-test for OPDA and water gave a T-stat of 0.15 and a 2 tailed p-value of 0.89. These results indicate that there is no difference in growth rates in the presence of chemicals.


JA1
JA2
y = 0.9214x - 1.2464
y = 0.8714x - 0.9214
Diameter of Growth (cm)
8
6
4
2
0
-2
8
16
22
32
40
51
60
72
Hours of Growth

A.


MeJA1
MeJA2
y = 0.8869x - 0.6286
y = 0.906x - 0.9393
Diameter of Growth (cm)
7
5.25
3.5
1.75
0
-1.75
8
16
22
32
40
51
60
72
Hours of Growth

B.



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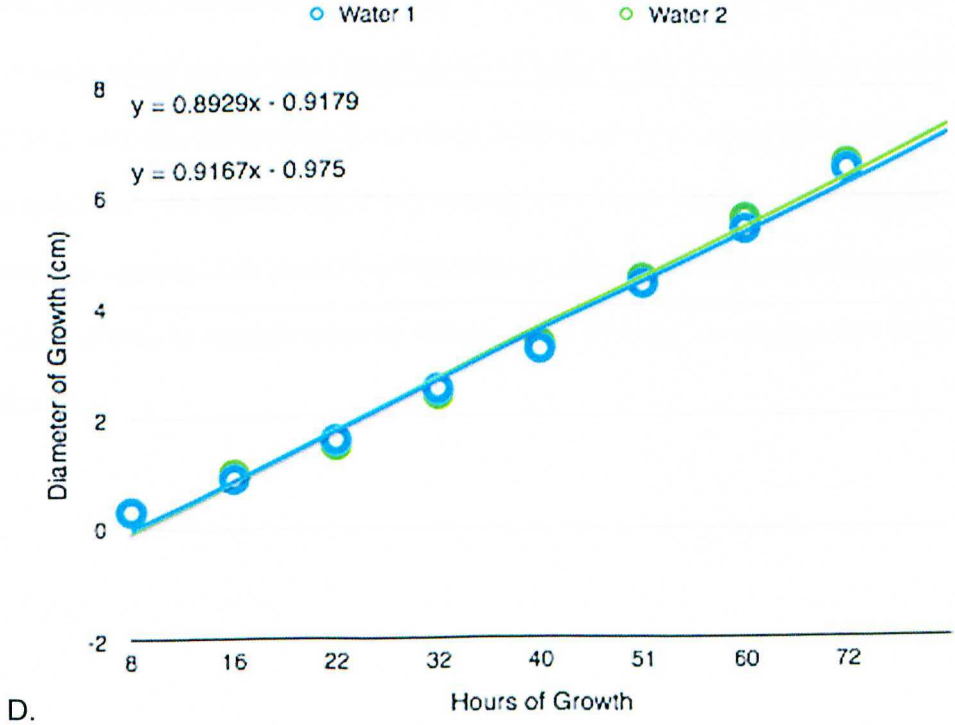

D.

Figure 5: *P. Irregulare* Growth Rates in Water versus in the Presence of Chemicals in the JA Pathway. The rate of growth of *P. irregulare* is shown for A) 100µL of 50µM JA, B) 100µL of 50µM MeJA, C) 100µL of 50µM OPDA, and D) 100µL of water.

Conclusion:

It was shown that application of JA, MeJA, or OPDA directly to the fungus had no effect on its growth. As shown in Figure 5, the growth rates for all treatments were very similar. This was confirmed through statistical analysis using a t-test with water as a control. The p-values were all above 0.05 and therefore there was no statistical difference between the growth rates in the presence of the hormone verses in the presence of water. This suggests that the any moss death observed was caused by the fungus killing the plant due to the fungus not being inhibited by the chemicals applied exogenously to the moss samples.

My results did not support the idea that JA can induce pathogen resistance in moss. My results were not what was expected given that Oliver (2009) found that JA induces defense gene expression. This experiment should be replicated in order to provide more conclusive evidence for or against what has previously been reported. JA has previously been shown to induce SAR in the moss (Loake and Grant 2007). After JA application, the moss should be resistant to fungal infection, showing that JA turned on defense genes. It is assumed that these genes are most likely PR genes, which have been shown to have enhanced expression following JA treatments (Tiryaki and Tunaz 2004).

Chapter 2: Gene Identification and Expression in *P. patens* Following JA or Fungal Elicitor, Chitosan

Introduction:

Plant pathogen interactions have led plants to evolve two forms of induced responses to provide the plant with resistance, HR and SAR. HR is a localized response that kills infected tissue to prevent the spread of the pathogen (Morel and Dangl 1997). SAR is a system that gives resistance to the entire plant following infection, which prevents future infection (Ryals 1996). These two induced defense systems are characterized by genes that are highly expressed when the plant is showing pathogen resistance. These defense genes that are often expressed are PR genes. PR proteins function as cell wall inhibitors, such as chitonases or β-glucanases (Ebrahim 2011). Another defense related gene that is often expressed is CHS. CHS functions as a flavonoid producer and prevents fungal infection (Cain 1997).

Once a pathogen has infected the plant, JA genes are expressed to assist in forming JA. JA genes, specifically AOC, AOS, and LOX, are important in forming compounds in the JA family, such as MeJA, OPDA, and JA. Once JA is formed, SAR and HR are initiated (Oliver 2009).

Genes that are related to the JA pathway have been identified in vascular plant species, but are not confirmed in nonvascular species. Using *Arabidopsis thaliana* as a model plant, similar genes were found in *P. patens* using NCBI BLAST. I expected that genes that encode enzymes in the JA pathway, such as AOC, AOS, and LOX, will be induced by pathogen exposure or exposure to a fungal like chemical, such as chitosan, in order to help elicit SAR in the moss. It is also thought that JA will turn on defense related genes, such as the PR genes, due to the fact that JA induces resistance in moss as shown in the previous experiment.

Methods:

Gene Identification

The publicly available DNA database search engine BLAST was used to identify moss genes that are involved in the production of JA and MeJa. Genes that I looked for include allene oxide synthase (AOS) and allene oxide cyclase (AOC), (two genes needed to produce OPDA), OPDA reductase (a gene needed to produce JA), and jasmonic acid carboxyl methyltransferase (a gene needed to produce MeJa). Once identified, PCR primers were produced for each gene, and these primers were used in quantitative RT-PCR (qRT-PCR) following inoculation with chitosan. I expected to find that the genes that regulate the JA pathway were turned on after pathogen infection. The amino acid sequences for *A. thaliana* and *P. patens* were compared using ClustalW. ClustalW compares the sequences and determines how similar they are based on the amino acid sequence.

Quantitative Real Time PCR (qRT-PCR) Shows JA Genes Turned on After Chitosan Application

qRT-PCR was employed to detect which genes were expressed after exogenous chitosan application on *P. patens.* 30 µL of chitosan (1mg/ml) was applied over the entire moss sample and after 1, 2, 4, or 24 hours, the mRNA was extracted using a RNA extraction kit (Qiagen). AOC, AOS, and LOX were examined for quantification of expression. These genes were compared to an Actin control that should be expressed universally in all areas of the plant at all times. Three replicates of each were performed.

qRT-PCR Shows Defense Genes are Turned on After JA Application

qRT-PCR was again performed after JA treatment on the moss. 30µL of 50 µM JA was

applied to the moss and after 1, 2, 4, and 24 hours the mRNA was extracted. qRT-PCR was performed focusing on the defense genes, PR1 and PR2. These genes were compared to an Actin control. Three replicates of each were performed.

Results:

Gene Identification Using BLAST

Based on the genes in *A. thaliana*, similar genes were found in *P. patens* using NCBI BLAST. The genes of interest that were found in *P. patens* using *A. thaliana* were AOC, AOS, and LOX. AOC was found in *A. thaliana* with an accession number of NP_189204.1 and a similar sequence, probably the AOC gene in *P. patens*, was found with an accession number of XP_001783824 (e-value of 5e-70). The ClustalW alignment (Figure 6) comparing the AOC gene sequences in *P. patens* and *A. thaliana* is shown. AOS was found in *A. thaliana* with an accession number of NP_199079.1. A similar gene was found in *P. patens*, that accession number is XP_001767870.1 (e-value of 4e-156). The ClustalW alignment comparing AOS between the two species is shown in Figure 7. Finally, LOX was found in *A. thaliana*, accession number of AAA32827.1, and using BLAST a similar sequence in *P. patens* was found, accession number of XP_001778088.1 (e-value of 0.0). The ClustalW alignment comparing LOX between the two species is shown in Figure 8.

JA Genes Expressed after Chitosan Treatment

qRT-PCR shows that AOC, AOS, and LOX are highly expressed after chitosan application. After chitosan treatment, all three genes are turned on. AOC is not expressed in moss originally or 1 hour after chitosan treatment, but after 2 hours the gene is expressed and expression increases until 4 hours after treatment. At its highest,

AOC has a 3.5 fold increase in expression at the 4 hour time point. AOS is highly expressed after 2 hours and is shown to be turning off at the 4 hour time point. Finally, LOX expression constantly increases through the 1, 2, and 4 hour treatments, reaching up to a 2 fold increase in expression at the 4 hour time point.

Defense Genes Expressed After JA Treatment

This portion of the experiment still needs to be completed. I expect to find that defense genes, such as PR1 and PR2, will be expressed following the JA treatment.

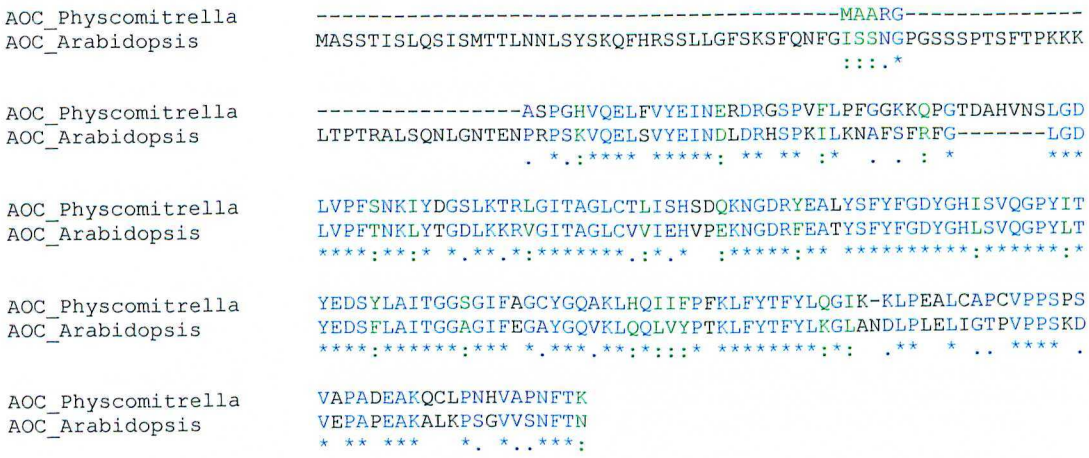

Figure 6: ClustalW Alignment of AOC Proteins in *P. patens* and *A. thaliana*. This figure shows the ClustalW alignment of the AOC protein using the amino acid sequences from *A. thaliana,* NP_189204.1, and *P. patens,* XP_001783824.

Figure 7: ClustalW Alignment of AOS Proteins in *P. patens* and *A. thaliana*. This figure shows the ClustalW alignment of the AOS protein using the amino acid sequences from *A. thaliana,* NP_199079.1, and *P, patens,* XP_001767870.1.

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LOX_Physcomitrella      MDRGLRQLLQSPPVLYEVESSLVDLKATIVLCKKEFFESSVAAEDGKVWDLEDELSGKRV
LOX_Arabidopsis         MFGELRDLLTGG----GNETTTKKVKGTVVLMKKNVLD--FNDFNASFLDRLHEFLGNKI
                        *   **:** .       *::  .:*.*:** **:.::  .   :... *  .*: *:::

LOX_Physcomitrella      YLQLVSNDVDSSTG--KAMRTSEMMIENWTESSTSSSHIASTYPTKFVVNFRVKKEFGEP
LOX_Arabidopsis         TLRLVSSDVTDSENGSKGKLGKAAHLEDWITTITS----LTAGESAFKVTFDYETDFGYP
                         *:***.** .* .  *.   .   :*:*  : **     ::  : * *.*  :.:** *

LOX_Physcomitrella      GALFVKNFHRNEFLLKEITVE-VPNRSSLHFICDSCVYNVDHYAADRAFFTNKVYLPRET
LOX_Arabidopsis         GAFLIRNSHFSEFLLKSLTLEDVPGHGRVHYICNSWIYPAKHYTTDRVFFSNKTYLPHET
                        **:::::* * .*****.:*:* **.:. :*:**:* :* ..**::**.**:**.***:**

LOX_Physcomitrella      PAGLQELREHLLQQLRGNGTGERKEADRIYDYHVYNDLGDSYRHDSLKRPVLGDSDEFPY
LOX_Arabidopsis         PATLLKYREEELVSLRGTGEGELKEWDRVYDYAYYNDLGVPPKNP---RPVLGGTQEYPY
                        ** * : **. * .***.* ** ** **:***  ***** . ::    *****.::*:**

LOX_Physcomitrella      PRRMRTGRQRSKTDPEAEDRGDLWT--NFYIPRDERYTMVKSEKLQEDAIQTASRKLLPA
LOX_Arabidopsis         PRRGRTGRKPTKEDPQTESRLPITSSLDIYVPRDERFGHLKMSDFLAYALKAIAQFIQPA
                        *** ****: :* **::*.*  : :  ::*:*****:  :* ..:   *::: :: : **

LOX_Physcomitrella      IQALYSR-QTEFESVREIADLFKKGVSLT-------VPSDSDIDLDSDRTTPECEIILTY
LOX_Arabidopsis         LEAVFDDTPKEFDSFEDVLKIYEEGIDLPNQALIDSIVKNIPLEMLKEIFRTDGQKFLKF
                        ::*::.   .**:*..:: .::::*:.*.       : .:  ::: .:   .: : :*.:

LOX_Physcomitrella      PTPKVIAVDETAWTMDEEFAREMLAGLNPVVIERLREFPIKSKLDEDTYGDPVSAITAEH
LOX_Arabidopsis         PVPQVIKEDKTAWRTDEEFAREMLAGLNPVVIQLLKEFPPKSKLDSESYGNQNSTITKSH
                        *.*:**  *:***  *****************: *:*** *****.::**:  *:** .*

LOX_Physcomitrella      IEPFLEDMDVRTALEGHKLFVLDYHDAFLPFVSKINENPACKAYATRTFLFLTHEGILRP
LOX_Arabidopsis         IEHNLDGLTVEEALEKERLFILDHHDTLMPYLGRVN-TTTTKTYASRTLLFLKDDGTLKP
                        **  *:.: *. *** .:**:**:**:::*::.::* ..: *:**:**:***..:* *:*

LOX_Physcomitrella      VAIEVSLPQESEP--GTVKRIFTPPPMGMKDWMWELAKVHVLANDAGYHQLSSHWLRSHA
LOX_Arabidopsis         LVIELSLPHPNGDKFGAVSEVYTPG-EGVYDSLWQLAKAFVGVNDSGNHQLISHWMQTHA
                        :.**:***: .    *:*..::**   *: * :*:***..* .**:* *** ***:::**

LOX_Physcomitrella      TMEPVIIATHRQLSTLHPIYQALVPHMKNTLDINAAARKALINAGGIIEMTFTPHAYAMQ
LOX_Arabidopsis         SIEPFVIATNRQLSVLHPVFKLLEPHFRDTMNINALARQILINGGGIFEITVFPSKYAME
                        ::**.:***:****.***::: * **:::*::*** **: ***.***:*:*. *  ***:

LOX_Physcomitrella      ISSAVYKQSWRFDEQALPTDLIKRGMAIPDKNGDNGLKLVIEDYPFATDGLELWGAINEW
LOX_Arabidopsis         MSSFIYKNHWTFPDQALPAELKKRGMAVEDPEAPHGLRLRIKDYPYAVDGLEVWYAIESW
                        :** :**: * * :****::* *****: * :. :**:* *:***:*.****:* **:.*

LOX_Physcomitrella      LREYVDLIYSDDGEVEEDRELQSWWYEIRYVGHGDKKDAEGWPTLNSKASLVHTLTTIVW
LOX_Arabidopsis         VRDYIFLFYKIEEDIQTDTELQAWWKEVREEGHGDKKSEPWWPKMQTREELVESCTIIIW
                        :*:*: *:*. : ::: * ***:** *:*  ******.   **.:::: .**.: * *:*

LOX_Physcomitrella      IASCHHAAVNFGQYEYAGFMPNHPTMTRRLIPMEGSPEFLELEQDPEAFYLSTISNETQA
LOX_Arabidopsis         VASALHAAVNFGQYPVAGYLPNRPTISRQYMPKENTPEFEELEKNPDKVFLKTITAQLQT
                        :**. *********  **::**:**::*: :* *.:*** ***::*: .:*.**: : *:

LOX_Physcomitrella      TVIMTTTEVLSTHSSHEEFLGQRSTPNWTSDEKISAVYGRFQERIGEIEELIKARNQEKR
LOX_Arabidopsis         LLGISLIEILSTHSSDEVYLGQRDSKEWAAEKEALEAFEKFGEKVKEIEKNIDERNDDET
                         : ::  *:******.* :****.: :*:::::   .: :* *:: ***: *. **:::

LOX_Physcomitrella      LKNRYGRVQLPYELLYPSSDHGLTGKGVPNSTSI
LOX_Arabidopsis         LKNRTGLVKMPYTLLFPSSEGGVTGRGIPNSVSI
                        **** * *::** **:***: *:**:*:***.**
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Figure 8: ClustalW Alignment of LOX Proteins in *P. patens* and *A. thaliana*. This figure shows the ClustalW alignment of the LOX protein using the amino acid sequences from *A. thaliana,* AAA32827.1, and *P, patens,* XP_001778088.1.

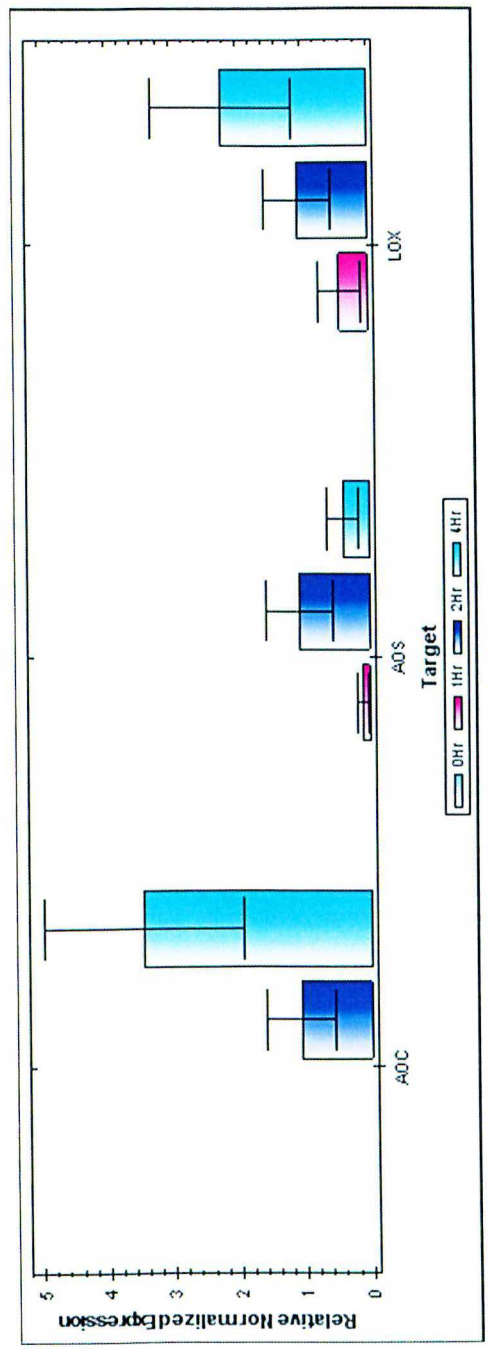

Figure 9: qRT-PCR Data Analyzing AOC, AOS, and LOX Gene Expression in *P. patens* 0, 1, 2, or 4 hours After Chitosan Treatment. After 2 and 4 hours AOC increases in expression 3.5 fold. AOS increases from 1 to 2 hours and then decreases in expression at the 4 hour time point. LOX increases expression steadily at the 1, 2, and 4 hour time points up to a over a 2 fold increase in expression. The bars represent standard deviation between the three replicates.

Conclusion:

I expected to find homologs of the JA genes in *P. patens* and that these genes are expressed in moss following treatment by the fungal elicitor, chitosan. When searching for the homologous genes in *P. patens,* I found good protein matches for each gene to *A. thaliana.* The matches had e-values close to zero, verifying their similarity. Both plant species have similar JA pathway proteins, suggesting that both vascular and non-vascular plants have similar JA synthesis pathways.

These genes that were found using BLAST were expressed after exogenous JA treatment. It was also shown that JA pathway genes, AOC, AOS, and LOX, were turned on after fungal cell wall product, chitosan, had been applied to the moss. The highest expression was found at 4 hours in both AOC and LOX. AOS had its highest expression 2 hours following chitosan treatment. My results are similar to what Oliver (2009) found when they found that fungus induced expression of defense genes. These genes are all used to produce JA products, such as MeJA, JA, and OPDA. By turning on the JA pathway, JA is produced which initiates SAR in the moss (Oliver 2009). Therefore, fungal infection initiates a cascade that turns on the HR and SAR. SAR will then protect the moss from future infections. This experiment needs to be replicated to confirm these results. Adding in additional time points to this experiment, such as 24 hour and 48 hours, would also be beneficial to gain a broader understanding of what is happening. These genes may be decreasing in expression after a longer amount of time because the plant does not need the defense genes expressed constantly. By utilizing more time points, it could be determined whether or not gene expression ever returns to the original level. This experiment needs to be conducted using *P. irregulare* instead of chitosan to verify that a pathogen gives similar results. In order to confirm that SAR is being initiated, I need to extract tissue from a site distal of the site of application of the JA or

chemical in the JA pathway. This would ensure that the genes are expressed in all areas of the plant and not only at the site of application.

I was not able to test whether or not JA induces expression of defense genes. In the future, I hope to see that JA turns on the defense genes, such as PR genes. In order to complete this experiment, better primers need to be identified for the defense genes in *P. patens*. This experiment would need to be replicated multiple times as well. These PR proteins are important in initiating SAR and HR in the plants by acting as cell wall disrupters. These proteins inhibit the growth of the pathogen, thereby protecting the plant from further infection. These proteins act locally and over the plant as a whole to help protect all areas of the plant against infection.

CONCLUSION

Despite inconclusive evidence regarding whether or not JA induces a systemic defense response, the gene experiments conducted suggest that the pathogen imitator, chitosan, induces JA production. JA is produced in response to pathogen infection and initiates a cascade response in the plant that induces resistance. Pathogen infection also increases expression of genes in the JA pathway such as LOX, AOC, and AOS. Each gene is turned on 1-2 hours following JA application and decreases in expression by 24 hours after JA application. These genes assist in turning on the SAR and HR responses. JA genes are used to produce JA, and once JA is produced PR genes are then expressed. This is similar to how vascular plants induce defenses as well (Loake and Grant 2007). It is assumed that these genes are functioning similarly in nonvascular plants and vascular plants. The PR proteins are responsible for inhibiting pathogen spread and killing all pathogens by lysing the cell wall and disrupting necessary functions.

Despite moss not having a vascular system, it has been shown that there is a way for the plant to transport signals across the plant. It has not been determined yet how those signals are transported. Further research needs to be completed to determine if there is a chemical signal that migrates through the plant to initiate an SAR response throughout the entire plant. It is assumed that the chemical that is migrating in vascular plants is SA (Ryals 1996). These signals are most likely similar in nonvascular plants, but more research must be completed to determine that definitively.

Once these plant defense systems are fully characterized, it will be understood how plants respond to pathogen infection. This will assist in determining the evolution of defenses, due to vascular and nonvascular plants having very similar defense systems.

There is much research to be completed through testing other plant hormones and determining the response that these have on nonvascular plants. Also, this research has been done on only one species of moss, and showing that this is universal through all nonvascular species will be important. This research can be used in farming to help protect crops from damage due to pathogens. Farmers will hopefully be able to better protect their crops once we know more about the plant-pathogen interactions involved.

We have shown that nonvascular moss is much more complex than originally expected. It has ways of eliciting responses that were thought to be impossible in plants without a traditional vascular system of xylem and phloem. This is an exciting field with many more possibilities for future research and practical value.

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